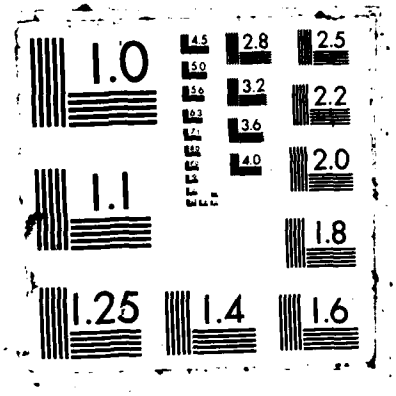
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CRYSTALLIZATION AND VITRIFICATION IN CRYOPROTECTED AQUEOUS SYSTEMS

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CRYSTALLIZATION AND VITRIFICATION IN CRYOPROTECTED AQUEOUS SYSTEMS

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SUMMARY

Some key ideas, and experimental findings concerning the probability that crystallization of a liquid or its binary solutions will occur at moderate cooling rates are discussed, with emphasis on the case of aqueous solutions. The use of cryoprotectants and of pressure to diminish these probabilities, hence to promote vitrification, is rationalized. Some new data on crystallization of bulk and emulsified aqueous solutions of the cryoprotectant glycerol are presented to illustrate the principles.

INTRODUCTION

In the search for improved methods of preserving multicellular systems and recovering them in viable condition, attention must be focussed on the avoidance, or at least the careful control, of crystallization of ice. Although it seems that there may be some aqueous systems from which ice could never crystallize (because the liquid enters the glassy state while it is still in the thermodynamically stable state - for instance $H_2O + H_2Cr_2O_7$ solutions of eutectic composition, (Vuillard, 1957)), such cases are not of great relevance to cryobiological practice. In most systems there is a close correlation between the ability of the solution to support living cells and the ease with which the solution generates ice crystals during cooling. This is no doubt due to the fact that it is the "free", or unbound, water which is involved in each function. The cryobiologist's task is to suppress the latter as far as possible without prejudicing, too much, the former. In this effort, the need to understand the rate of ice nucleation and rate of growth of ice crystals in relation to temperature, pressure, and composition variables, is obvious.

When ice fails to form during cooling, then a *homogeneous* glassy state of the solution will usually, but not always, be produced on sufficient cooling. Sometimes the solution will split into *two distinct* liquid phases which will each then vitrify - in fact one of the principal cryoprotectant systems, PPG + H_2O seems to behave in this surprising manner (MacFarlane (1986), Vassoille et al (1986), Boehm et al (1987)) which may be of significance to understanding its favorable properties. In such a system, cell integrity is assured when the system remains cold. However, in many cases,

such vitrified solutions are predisposed to generate ice crystals (sometimes explosively) on reheating. Such events clearly must be under the control of the cryobiologist or cells will be destroyed in the attempt to recover them from cold storage (MacFarlane, 1986).

It is the aim of this article to present the central ideas and experimental observations relevant to ice nucleation and growth in aqueous solutions during cooling, in terms which can be digested and utilized by the non-expert.

In order to understand the processes of, and competition between, crystallization and vitrification in aqueous systems it is necessary to understand both the driving force which leads a supercooled liquid towards crystallization, and the kinetic factors which may frustrate the realization of the crystalline state.

The driving force unfortunately cannot be appreciated without some knowledge of thermodynamics, and cannot be correctly represented in other terms. We will refer to this driving force as the "free" energy difference G(crystal)-G(liquid)and give here a brief review of its nature. Stability in Nature can be either thermodynamic or kinetic in origin. It is generally known that glasses are not stable states with respect to crystals of the same composition, yet glasses have existed on the moon's surface for at least half the age of the solar system. Such stability is kinetic in nature and reflects the intrinsic slowness of large scale molecular reorganization in vitreous materials at temperatures far below their "glass transition" temperatures - indeed it is this sort of stability we seek to bestow on the liquids in and around the multicellular systems of interest to this meeting. Thermodynamic stability, on the other hand, is more subtle. It requires that the state which is stable be selected in the face of free access to all other possibilities. Crystallization is the process of achieving the thermodynamically stable state from an initially liquid state as the temperature falls below the freezing point.

The stable state of water molecules at one atmosphere pressure and 25^{o}C is not the lowest energy state, since ice (ice Ih) provides energetically the most favorable structural arrangement of water molecules at one atmosphere pressure. The ice structure is energetically favored over the liquid water structure by 44 kJ for each mole of water molecules. The melting of ice at 0^{o}C reflects not merely the thermal disruption of this ice lattice. Rather, and more profoundly, it reflects that some other directing force in Nature has taken control. Reversing the direction, we see that the increasing driving force to crystallization, which occurs as water is supercooled, reflects the decreasing importance of this other "directing force" with respect to the energy advantage of organization into the crystal. It is necessary therefore, to remind ourselves of the origin of the other directing force. Its essence lies in disorder and its power comes from the fact that disordered states are intrinsically more probably in nature simply because there are more ways of arranging molecules in messy arrangements (liquids or gases) than in neat ones (crystals). The natural tendency to disorder, hence to liquids rather than crystals, is overcome only if there is a substantial energy penalty to pay to disorganize; when the energy advantage of the crystal is not large, the substance vitrifies easily.

The degree of disorder characterizing a collection of molecules, whether it is vibrational disorder in a crystal, or positional disorder in a liquid, can be measured in the laboratory, and is given the symbol S. Naturally, S is larger for liquids than for solids of the same composition.

The dimensions of S are such that the product TS is an energy and most importantly, an energy which increases with increasing temperature. It is this "disorder energy" which, in combination with the binding energy of the molecules, H,

determines the competitive status of one state of organization of matter, e.g., liquid, with respect to other states, e.g., crystal or gas. We express this balance of energy components by the term "free energy" G and write G = H-TS. Nature always seeks the state with lowest (most negative) free energy G.* We can now understand how the driving force to crystallization, which reflects the increasing dominance of binding energy over disorder energy, builds up as T falls below the melting point by graphing G for liquid and crystal states, as in Fig. 1. The G curves cross at the melting point T_m. Above T_m the disorder energy predominates, and the liquid (large S value therefore larger slope of G vs T) is stable due to the larger S value. Boiling reflects the crossing of the liquid and gas G curves, see Fig. 1.

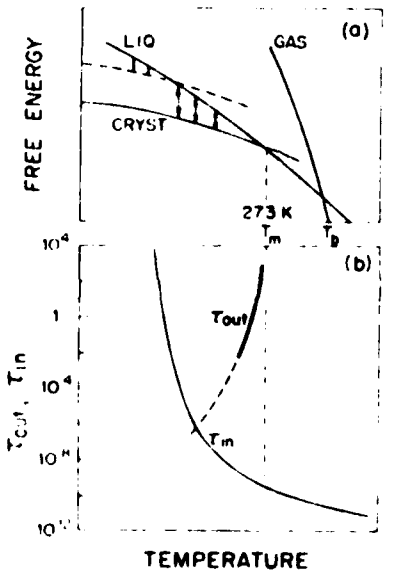


Figure 1. (a) Free energy vs. temperature curves for gas, liquid and two crystalline states for a substance with the freezing point of water, illustrting build-up of thermodynamic driving force for crystallization with increasing supercooling. (b) Variation with temperature of characteristic times for internal relaxation in the liquid state τ_{in}, and for crystallization of fixed volume fraction of the supercooled liquid τ_{out}.

We have drawn the curves in Fig. 1 with ice and water in mind, ignoring for the moment the evidence for an impending catastrophe at -45°C, Speedy and Angell (1976) and Speedy (1982), and instead treating water as if it were one of the more common molecular liquids about which somewhat more is understood.

The double arrows in Figure 1 shows how the driving force to crystallization, ΔG in Figure 1, builds up as T decreases below 273 K.

We must now recognize two time scales in our problem. The first is the time scale for crystallization of the liquid sample as *T* falls below 273 K on cooling. This time, considered as the time needed for a chosen volume fraction of the sample to

*We set the zero point for G as the energy of the dilute gas, i.e., infinite separation of molecules) at T = 0. Thus H is always negative (more so for crystals than for liquids) and becomes more negative with increasing temperature.

We have included in the diagram a second crystalline ice curve which lies always above the curve for ice I_h. This is to indicate the existence of the unstable polymorph ice I_c which is often the product of devitrification of water-containing systems. The diagram shows that below a certain temperature, T << 273 K, the liquid is unstable with respect to ice I_c so that its formation from the strongly supercooled liquid is not surprising. It is merely another example of the Ostwald step rule which says that when a metastable state breaks down, the system usually moves to the nearest free energy minimum, not the lowest one, because it is usually the nearest

become crystalline, initially decreases with decreasing temperature as the driving force ($G_c - G_l$) shown in Fig. 1 builds up. We call this time the escape time, τ_{out} and show it as a heavy line in Fig. 1(b). The second time scale which we denote τ_{in} is the time scale for relaxation *within* the supercooling liquid. τ_{in} increases continuously as the temperature goes down and, provided crystallization does not occur, becomes of the order of hundreds of seconds when the temperature enters the range of 140-160 K depending on what cryoprotectant may have been added. It is the arrival of this internal relaxation time at large values which causes the glass "transition" at T_g. (The transition reflects the inability of the liquid structure to adjust to the changing temperature for $T < T_g$ because of this lengthening equilibration time.) To give some structural significance to τ_{out} we note that it reflects a complicated combination of (a) the time necessary for one or more embryonic crystal with the same molecular organization as in ice I, to form (by a chance fluctuation in the positions of a large number of water molecules in the liquid, i.e. nucleation) and (b) the time necessary for these to grow spontaneously (by transfer across the liquid-nucleus surface of further water molecules, i.e. growth) until the chosen fraction, here 50%, of the liquid has transformed.

If the time scale for crystallization were actually to intersect the internal relaxation time curve, then vitrification would be intrinsically impossible. This is because below the intersection temperature the system would always move more rapidly towards the more stable crystalline state, (see Figure 1(b), dashed line). Fortunately, τ_{out} does not decrease continuously but instead enters a regime where its value is controlled by the same liquid diffusion processes which determine the behavior of τ_{in}. This regime exists because of the need for nuclei, once formed, to grow in order for a measurable fraction of the sample to become crystalline. Even for the nucleation process itself, there is a temperature of maximum formation rate (which occurs at temperatures below that of the τ_{out} nose). This maximum occurs because even the growth of a crystal embryo to a size sufficient to overcome its excess surface free energy and become thermodynamically stable, is diffusion-controlled, (Turnbull and Fisher, 1949). The result is (Fig. 2(a)) that the escape time τ_{out} exhibits a minimum value, designated τ_{nose}, and it is the job of the cryobiologist to ensure either that the time characterizing the nose is made long (so that it can be bypassed during relatively slow cooling) or that a cooling process which is fast with respect to the minimum crystallization time is developed. Note that the τ_{out} curves of Figs. 2(a) and 2(b) are just the familiar time-temperature-transformation (TTT) curves of nucleation and growth theory (Uhlmann, 1972) turned on their ends. In the following we discuss several strategies for changing the value of τ_{nose}, and, alternatively, developing methods of cooling fast enough to bypass it The latter problem is discussed in detail by Mayer, 1986.

We note first, however, that the actual value of τ_{nose} for the crystallization of pure water is not known, although several estimates for the value of the cooling rate needed to avoid "homogeneous" nucleation have been made. They range from 10^{-6}-10^{-10} sec, Sargeant and Roy (1968), Turnbull (1969), Fletcher (1971) and Uhlmann (1972). These values indicate the magnitude of the problem of preventing crystallization of water, and it should be noted that the crystallization of bulk samples will occur even more readily since, in bulk samples, crystallization is nucleated "heterogeneously," i.e the crystallization process commences on a extraneous surface which is almost inevitably present in bulk samples, [for review see Franks (1982)]

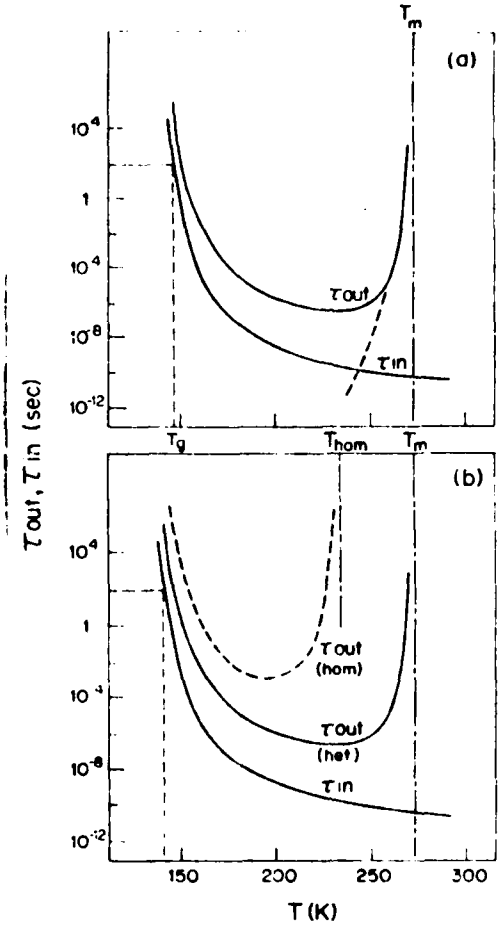


Figure 2. Relationship between escape time, τ_{out}, and internal relaxation time, τ_{in}, for (a) heterogeneously and (b) homogeneously nucleating liquid systems.

VITRIFICATION BY RAISING τ_{nose}

1. Increasing τ_{nose} by suppression of heterogeneous nucleation.

The first strategy we might explore is to guarantee that the sample, when it crystallizes, must do so by intrinsic fluctuations rather than by taking advantage of foreign surfaces to catalyze the process (heterogeneous nucleation). Since the number of dust particles per cc of aqueous solution is limited, the heterogeneous process can be made less probable by subdivision of the sample. This can most conveniently be accomplished by emulsification. Numerous studies of the crystallization of water in emulsions have been reported, Rasmussen and McKenzie (1972), Clausse et al. (1974), Broto, et al. (1976), and Kanno and Angell (1977), and many surfactants and inert matrix phases prove satisfactory. Figure 2(b) shows the effect of suppression of heterogeneous nucleation on the position of τ_{nose}.

That emulsification usually, though not always, leads to homogeneous nucleation has been demonstrated by comparisons of the crystallization temperatures of various molecular liquids in aqueous matrix emulsions with the directly measured nucleation temperatures based on cloud chamber microdroplet experiments, MacFarlane and Angell (1981).

2 Increasing τ_{nose} by addition of cryoprotectants: the examples of LiCl and glycerol.

A very effective strategy for increasing the value of τ_{nose} is to add a second component to the water. Such a component, when compatible with tissues, is called a

particularly convenient for experimental studies of the crystallization phenomenon, e.g. LiCl, which can be added in variable quantities without changing T_g, (Angell et al., 1982), hence without much affecting the viscosity or τ_{in}.

The reason why second components are beneficial in this respect is a simple one. The free energy of the water in the liquid state is always depressed when water is diluted with other molecules, whereas the free energy of ice which will crystallize is, of course, unchanged so long as the second component is not incorporated in the ice lattice. Since the ice lattice is extremely particular about incorporation of impurities, it is a general rule that the ice free energy will be unchanged. This means that the temperature at which ice formation can commence, i.e. the intersection temperature for G_{ice} with $\bar{G}_{water}$, is decreased, see Fig. 3. Assuming the viscosity of the solution is unchanged (implying τ_{in} is unchanged) this circumstance squashes the τ_{out} curve towards the τ_{in} curve with the result that the minimum is forced to occur at longer times (see Fig. 3(b)).

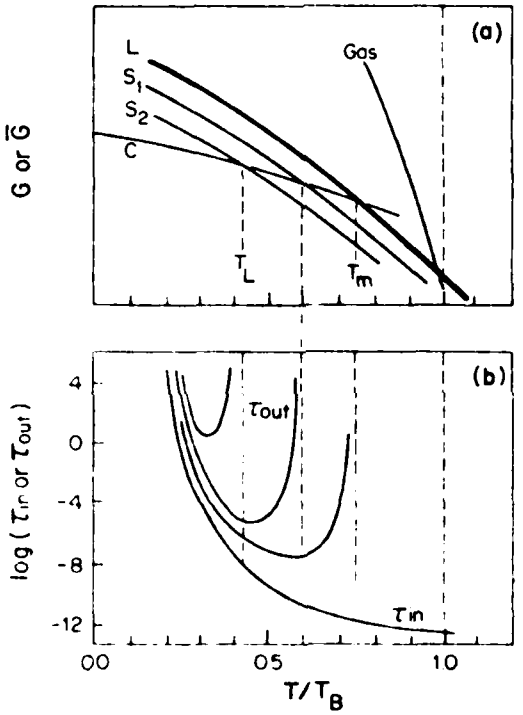


Figure 3. (a) Thermodynamic relations affecting crystallization of solutions compared with pure liquids, showing how adding a second component can lead to satisfaction of the $T_b/T_i \geq 2$ rule for the crystalizing component. (b) Effect of solution thermodynamics on the τ_{out} vs τ_{in} relations at constant viscosity, to explain the enhanced glassforming properties of solutions over pure liquids.

If enough solute is added to the water, the kinetics of crystallization may be reduced sufficiently that direct experimental determinations of the τ_{out} curve can be performed. A simple technique is that of step crystallization calorimetry, using a small sample differential scanning calorimeter (Perkin Elmer DSC-4) and its results have been described in several articles, (Angell and MacFarlane, 1981; MacFarlane et al., 1983a, b; Kadiyala and Angell, 1984). In Fig. 4 we reproduce the results obtained in the author's laboratory for crystallization of several solutions of lithium chloride in water, and give new data by the present authors for the more relevant system, glycerol + water.

Each point used to define the τ_{out} curves in Fig. 4 is the result of an experiment in which a small encapsulated sample of solution is cooled *suddenly* from the stable solution region to a chosen temperature below the liquidus and held there until the crystallization event is manifested by isothermal release of the heat of crystallization, see

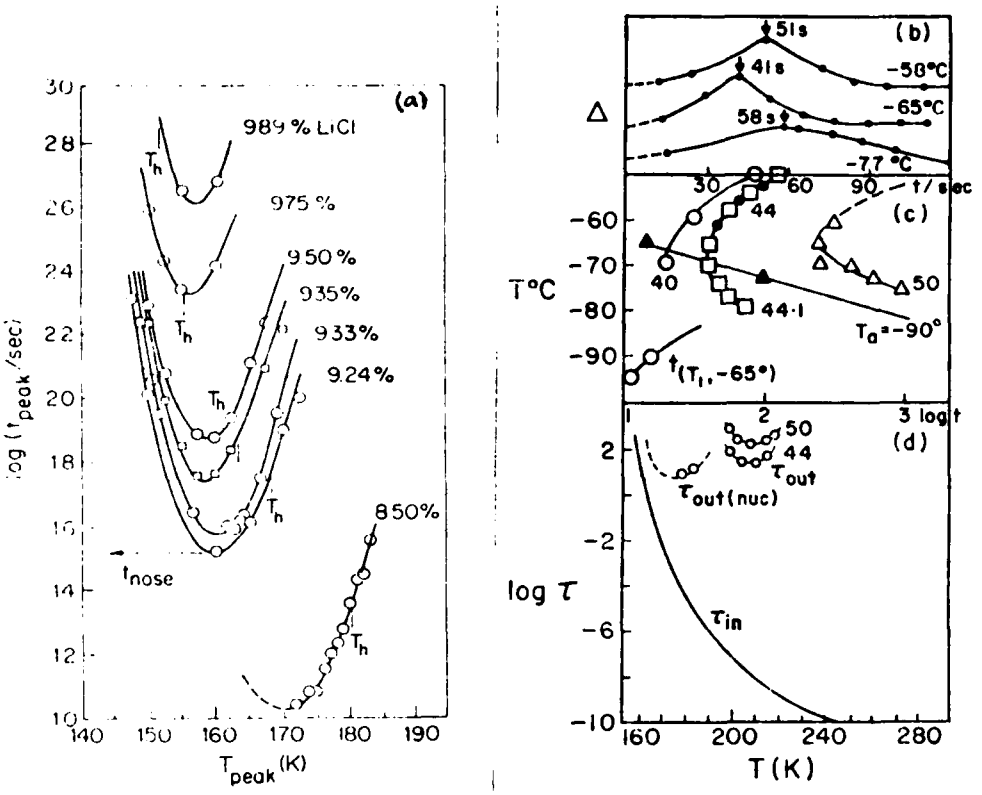


Figure 4. (a) Experimental determinations of τ_{out} curves for a series of emulsified LiCl + H_2O solutions showing the sensitivity of behavior like that of Fig. 3 to increasing concentration of the second component. The temperatures marked T_h on each curve are the temperatures at which crystallization is observed suddenly to commence on continuous cooling at 10°C/min. The filled circles are data from an unemulsified solution proving that, for this composition range, the crystallization rates are dominated by a homogeneous process. (b) Isothermal crystallization curves at three different temperatures for 44% glycerol-in-water solution from crystallization calorimetry experiments. (c) Classical time-temperature-transformation (TTT) curves constructed from the peak times of Fig. 4(b) type results, for three different bulk glycerol + water solutions, as marked. Note how nose of TTT curve moves to higher temperature as well as longer times as glycerol content increases, due to increasing viscosity. The two solid triangles are peak times for samples exposed to a 60 s nucleation at -90°C before holding at the crystallization temperature. The implied form of the nucleation curve from such two step experiments is indicated by the curve marked "nucleated". (d) τ_{out} vs. T representation of the same data as in Fig. 4(c). Some dielectric relaxation times for the solution of 50% glycerol are plotted as a dashed line to represent the values of τ_{in} for one solution in this system, to show the relation of experimental data to the schematic of Fig. 3.

Fig. 4b. The peak value of ΔE (the difference in instrument energy input to the sample and reference which is needed to maintain the set temperature) corresponds to the maximum rate of crystallization. A theoretical analysis (MacFarlane et al 1983a) based on the Avrami theory (Avrami 1939,1941) suggests that, for crystallization of solutions as in the present case, the peak value is reached when 45% of the water that will crystallise has crystallized. The time taken to reach the peak t_p of the heat release plot is recorded as a point of the τ_{out} curve as in Fig. 3b for glycerol + water solutions.

Comparison of Fig. 4a and 4d shows that while increase of solute concentration in each case results in the "nose" of τ_{out} curve being pushed to longer times, the temperature at which the nose occurs varies with solute concentration in opposite directions for the two solutes considered. This is because the solutes have different effects on the solution viscosity which controls the growth rate of nucleated crystals. In the case of LiCl, the viscosity is little affected by the salt addition, and the glass transition

rapidly. Another distinction lies in the composition dependence of t_{nose} which is very much greater in the case of LiCl solutions due to the fact that each Li^+ ion added coordinates 4 to 6 water molecules directly and effectively withdraws a total of 6 to 7 from the water structure (Angell and Sare, 1970).

The τ_{out} curves seen in Fig. 3 represent the composite effect of sequential nucleation and growth processes. A differential scanning calorimetry technique for assessing the relative importance of these two processes on the overall crystallization kinetics has recently been described (Kadiyala and Angell, 1984). The latter measurements have shown very directly how the upper part of the TTT curve is entirely determined by the nucleation rate (indeed the undercooling/surface tension- controlled part of the nucleation curve) while the lower part is dominated by the liquid transport-controlled crystal growth rate.

It is a general finding for molecular liquids that those with melting points less than half the boiling points (in K) vitrify in bulk with moderate cooling rates, (Turnbull and Cohen, 1958). The explanation for the rule is, essentially, that the boiling point determines the position of the τ_{in} curve, and the melting point fixed the origin for τ_{out}, (Angell et al., 1985).

Locating the origin for τ_{out} at $\frac{1}{2}T_b$ or less is thus the condition for pushing τ_{out} sufficiently towards τ_{in} for the nose to be squeezed out to long τ. The second component effect can thus be viewed as a device for rectifying the unfavorable T_b/T_m ratio of pure water. As it turns out, the glass-forming composition range for water seems to be reached when $373/T_f$ (T_f is the freezing temperature or, more correctly, the *liquidus* temperature at which ice *should* start to form on slow cooling) has increased to only 1.7, but this is consistent with the generally lower T_b/T_m values needed for vitrification in the case of hydrogen bonded liquids, (Angell et al., 1985).

3. Increasing τ_{nose} by increase of pressure.

Although direct measurements of the τ_{out} curve in response to changes in pressure have not yet been performed, the behavior can be predicted from the results of homogeneous nucleation temperature as a function of pressure. These have been reported for pure water and various aqueous solutions (Kanno et al., 1975; Xans and Barnaud, 1975). The homogeneous nucleation (defined earlier) *temperature* is the temperature at which, during continuous cooling, a liquid which is protected from heterogeneous nucleation suddenly commences to crystallize. It usually corresponds to a point on the high temperature branch of the τ_{out} curve, and the movement of the τ_{out} curve in response to either composition or pressure change can be implied from the behavior of T_h. When τ_{nose} has become long and crystallization can almost be bypassed at moderate cooling rates, Fig. 4 shows that T_h has moved around the τ_{out} curve to about the temperature of the 'nose' (i.e. the minimum in τ_{out}). We use these observations in the construction of Fig. 5(b).

The variation of T_h found by MacFarlane et al (1981) for pressure increases on two different cryoprotectant solutions (identified in the figure caption) are shown in Fig. 5(a) and the behavior of τ_{out} with P deduced from these results is shown in Figure 5(b). It is notable that in one of these no crystallization was observed, even though the concentration of cryoprotectants was in the range where no tissue damage is encountered. The development of fast quenches under high pressure by Moor (1986) permits τ_{nose} to be bypassed at relatively small, or zero cryoprotectant concentrations.

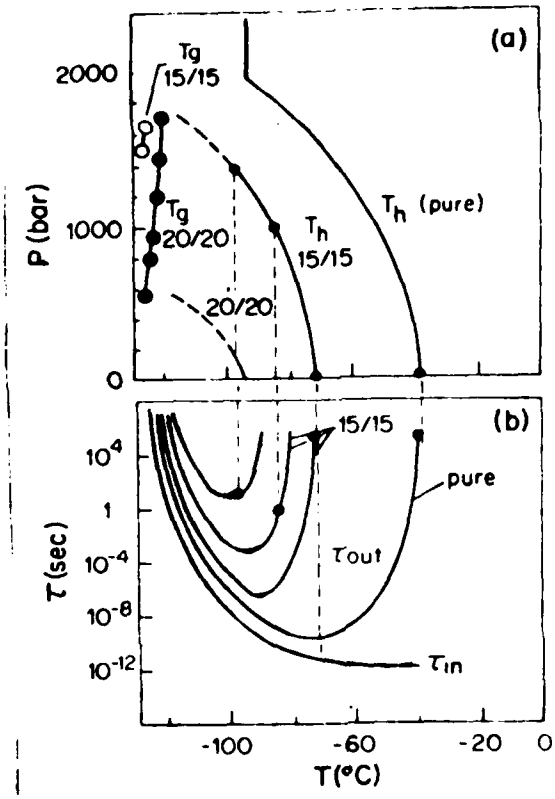


Figure 5. Experimental determination of the homogeneous nucleation temperature determined during continuous cooling of emulsion samples, as a function of applied pressure. Highest temperature curve is for pure water, the others are for water + 15 vol% propylene glycol PG+15 vol% dimethylsulphoxide DMSO, and 20% PG, 20% DMSO respectively. Glass-forming regions at high pressure are indicated by T_g data points (see MacFarlane & Angell, 1982).

4. Increasing τ_{nose} by reduction of sample size.

Although this section is of little relevance to cryopreservation technology because of the predetermined size of the sample to be preserved, we include some comments on the sample size effect because of the intrinsic interest content of the matter, and for the sake of completeness.

There are at least two ways in which reducing the size of the sample under study can affect the nucleation probability. The first, reduction in the statistical probability of the sample containing a heterogeneous nucleus, has been mentioned earlier. The second is that the probability of an entropy fluctuation of the magnitude necessary to produce a viable nucleus is reduced. The relative magnitudes of these effects is illustrated for the case of pure water by Fig. 1 of Mason (1958), but is also implied by the observation of Aguerd et al. (1982) that ordinary (~5 μm droplets) emulsions of water containing AgI seeds in concentrations such that every microdroplet has many heterogeneous nuclei, still supercool to -20°C (c.f. -4°C for bulk samples).

The small sample effect can be greatly magnified if the sample size can be made nanoscopic rather than microscopic as in emulsions. A possibility recently illustrated for molecular liquids (Angell et al. 1984b) is to form a *microemulsion* (ME) of the liquid of interest, in which the droplet size is reduced to the order of 5-10 nm. In such circumstances, provided the ME either remains stable to low temperatures, or is slow to separate, even very simple liquids such as CCl_4 and benzene can be vitrified (Dubochet et al. 1984).

MEs in which *water* is the dispersed phase are less simple to stabilize against

growing droplets. Preliminary evidence that this can be achieved was presented in an earlier version of this article (Angell and Choi, 1986) but as the subject has not been further developed since that time we will not discuss this aspect of water vitrification any further here.

5. Increasing τ_{nose} by micro-interference with the nucleation or the growth processes.

All the above factors for decreasing the probability of nucleation and growth of crystals during cooling of aqueous samples have been based on thermodynamic manipulation of the relative values of τ_{out} and τ_{in}. Except for the last mentioned approach (section 4) using sample size manipulation (which is impractical for organ preservation technology) the very same variation of conditions (cryoprotectant concentration, pressure) which discourages crystallization produces a parallel discouragement to cell survival due to causes not involving crystallization (i.e. due to lethal concentration, lethal pressures). This is presumably because both - cell survival and ice nucleation - require the presence of "water-like" (hence more or less ice I_h-like) arrangements of water molecules in the solution state. There is one possible way around this "catch 22" situation which merits discussion, and which has been the subject of exploratory studies by Duman (1982) and Knight and Duman (1986) MacFarlane et al (1986) among others. This involves the introduction of small quantities of exotic compounds which interfere in a non-thermodynamic manner with the nucleation and/or growth processes.

We imagine a molecular Maxwell's demon which is constructed in such a specific manner that it recognizes, and interacts selectively with, clusters of water molecules which have ice-like characteristics (i.e. are potential nuclei) and discourages them from growing, either by "capping off" their growth sites, or by distorting the complex away from "icelikeness". Because nucleation events are believed to be rare in time and space under most conditions a relatively small concentration of such "guardian" molecules could protect a large number of water molecules from nucleation events. Even if inhibition of crystallization occurred by the mechanism of "guardian" molecules preferentially absorbing on the surface of *viable* nuclei so as to slow down or arrest the crystal growth, the required concentration could be smaller than bulk concentrations by many orders of magnitude. Indeed, this is believed by some to be the mode of operation of the glycoprotein molecules which protect supercooled Antarctic fish from freezing (DeVries, 1971). With this idea in mind, MacFarlane and coworkers (1986), have synthesised water-soluble polymers for use in cryoprotectant systems.

The notion that fish glycoproteins could serve as cryoprotectants has been tested by Petzel and Devries (1979) but found impractical because of glycoprotein-induced cell damage. However, the concept seems a viable one, at least for moderate extensions of the supercooled regime, and for crystal-size refinement in frozen tissues, (D. Thursman, private communication) and deserves more attention by cryobiologists. There are, for instance cold climate insects which also contain thermal hysteresis proteins but which are freeze-tolerant i.e. can survive extracellular freezing (Duman 1982, Knight and Duman 1986), and the cryoprotective substances in freeze-hardy trees and plants remain to be elucidated.

VITRIFICATION BY QUENCHING FAST WITH RESPECT TO τ_{nose}.

The alternative strategy for avoiding crystallization of ice during cooling in the face of a very short ($<10^{-6}$ sec) τ_{out} is to reduce drastically the length of time the sample resides at or near the temperature of τ_{nose}, by raising the cooling rate. Long considered outside practical possibility, these cooling rates (no doubt aided by small sample effects, discussed above) have recently been achieved. Brugeller and Mayer (1980, 1982), using jet quenching of emulsions or sprayed droplets and Dubochet et al

(1981, 1982) using liquid ethane quenching of ~0.1μm films, and most recently Mayer (1986) have reported strong structural and thermochemical evidence for the vitrification of their samples.

Their methods are discussed elsewhere (Mayer, 1986,) and no attempt will be made to review them in this article. The evaluation of critical cooling rates for any liquid using the theory of nucleation and growth has been given by Uhlmann (1972) and, by more exact methods, in a recent paper by MacFarlane (1982).

A complication in the discussion of nucleation and growth theory for *pure* water (which is not of concern at most cryprotectant concentrations discussed so far) is raised by the existence of anomalous variations in the physical properties of the supercooled liquid which enter the theoretical expressions. For instance, an implication of the exponentially increasing heat capacity of pure water in the temperature range where nucleation is imminent (Angell et al., 1982) is that the rate of increase of the driving force G_L-G_C [Figure 1(a)] is abnormally small for water. The behavior of the liquid-solid surface free energy is currently unknown, but experimentally determined nucleation rates in this region (Taborek 1985) show no anomalies like those of most physical properties, so presumably there are mutually canceling effects. The region of diffusion control over τ_{out} lies at temperatures below the nose, so the influence of anomalous increases in viscosity (Speedy and Angell, 1976) cannot be observed.

CONCLUDING REMARKS

The challenge to gain control over the freezing process, one of the more common of nature's phenomena, is an exciting one. To meet such a challenge will require the development of new understanding in thermodynamics, kinetics, and structural aspects of the nucleation and growth processes and the nature of aqueous solutions, as well as a clarification of the precise mechanisms by which ice formation in cells causes loss of cell viability. The societal benefits of success in this endeavor can hardly be overestimated.

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